Dexrazoxane does not protect against doxorubicin-induced damage in young rats

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THE ANTICANCER AGENT DOXORUBICIN (DOX; Adriamycin; Adria) can cause life-threatening cardiac damage (8, 17). DOX-induced cardiac damage can be detected during or shortly after cancer treatment (early cardiotoxicity) or more than a year later (late cardiotoxicity). In adults, DOX cardiotoxicity is reduced by slow infusion of DOX and the use of limited doses. DOX cardiotoxicity can also be reduced by prior injection of the intracellular iron chelator dexrazoxane (DXR; ICRF-187; Zinecard; Pharmacia) (36, 37, 46). DOX-induced cardiotoxicity is due to DOX-mediated increases in oxidative stress causing apoptosis and not its ability to bind to DNA topoisomerase II cleavable complexes (40). DXR, with its higher affinity for iron than DOX, reduces DOX-Fe3+ complex formation, thereby lowering DOX-induced oxidative stress.

DOX is widely used in combination cocktails for treatment of childhood cancers. Like adults, children with DOX-induced cardiotoxicity develop early and late cardiotoxicities (10, 13, 26, 30, 32, 38). Some (11, 13, 26, 28), but not all (9, 25) studies suggest that young females are at greater risk for DOX-induced cardiotoxicity than young males. Childhood cancer survivors show a greater cardiac functional deficit and below-predicted exercise capacity, with females more affected than similarly treated males (14, 21, 29, 39, 42, 43). DXR is not recommended in the guidelines for treatment of pediatric malignancies (15).

In the present study, we injected DOX-alone, DXR-alone, or DXR + DOX into male and female day 10 neonate rat pups and assessed the ability of DXR to reduce the incidence of DOX cardiotoxicity. Day 10 neonate rats are somewhat similar to young children in that like children, the rat cardiomyocytes are completely differentiated and will not increase further in number, and the rats are actively growing in size.

MATERIALS AND METHODS

Materials. DOX and DXR were purchased from Sir Mortimer B. Davis–Jewish General Hospital Pharmacy. Primary and secondary rabbit antibodies and chemiluminescent detection kits were obtained from Santa Cruz Biotechnology or Pierce unless otherwise noted.

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Animal manipulation. All animal experiments were performed according to the guidelines of the Canadian Council on Animal Care. Lactating Sprague-Dawley dams with 15 pups per female were purchased from Charles River. At neonate day 10, rat pups were randomly divided into eight groups of at least five per gender. Pups were injected once intraperitoneally with either saline or drugs (DOX (3 mg/kg), DXR (60 mg/kg) or with DXR + DOX (60 mg + 3 mg) to result in a 20:1 DXR/DOX ratio). DXR was injected 30 min before the DOX injection. This dose of DXR and regimen reduced DOX cardiotoxicity in adult rats, mice, and dogs (18). After injection, rat pups were returned to their mothers until weaning on neonate day 22. Once weaned, all rats were fed standard rat chow. Rats were weighed before injection and daily, thereafter. Hearts were weighed at death and the heart weight-to-body weight (heart wt/body wt) ratio was calculated. Figure 1 contains a schematic diagram detailing the timing of experimental manipulations and death.

Exercise training. After weaning, rats were randomly divided into two groups; one set was left as is and the second set underwent a 2-wk swim training program. Rats swam in a 90 cm × 60 cm × 25 cm tub of 34°C water (5). Swimming started on day 14 postinjection (PI) with 2 × 5 min swimming periods separated by a 5-min rest period. Swim time was gradually increased to 3 × 15 min by day 21 PI and continued until day 28 PI. Thus rats were gradually acclimated to swimming and no rats were exhausted at the end of the swim periods.

Western blots. Hearts were homogenized in buffer (100 mM Tris pH 7.8, 200 mM NaCl, 0.5% Triton X-100 and protease inhibitors) using a Polytron, and the clarified supernatant was collected by a 15-min centrifugation at high speed in an Eppendorf centrifuge in the cold. Protein was quantitated by the Bio-Rad Bradford colorimetric method according to the manufacturers instructions. Proteins (40 μg) were separated through 13% SDS-PAGE gels, transferred to Immobilon-P membranes, and Western blots were performed by standard methods. Briefly, membranes were blocked in 10 mM sodium phosphate (pH 7.4) 137 mM NaCl, 3 mM KCl (PBS)/0.05% Tween 20/5% skim milk, incubated with primary antibodies (diluted 1:200 to 1:500), horseradish peroxidase-complexed secondary antibody (diluted 1:20,000) with interactions revealed by incubation with chemiluminescence substrates and exposure to X-ray film.

Protein carbonyl assay. Protein carbonyl assays were performed essentially as previously described (2). Protein, 20 μg, was derivatized with 2 M dinitrophenyl (DNP) hydrazine (DNPH) in HCl or HCl alone (control reaction), collected by TCA precipitation and resuspended in loading buffer. Solubilized proteins, 2 μg, were separated by SDS-PAGE and transferred to membranes for Western blotting by using anti-DNP (Sigma) antibody as described above. To verify equal protein loading, aliquots of the precipitated proteins were electrophoresed through SDS-PAGE and stained with Coomassie brilliant blue R-250.

Histological analysis. Hearts from all animals were cut in cross section at midheart, fixed in 4% buffered formalin, and processed routinely. Testes and ovaries were sliced longitudinally and in cross section, respectively. Sections were cut at 4 μm and stained with hematoxylin and eosin or Masson’s trichrome. Coded slides were examined by light microscopy with the code only revealed after completion of the examination.

Quantitation. Exposed films from Western blot analyses were scanned, and the areas under the peaks were quantitated by using National Institutes of Health Image 1.54 software. Statistical analysis for Western blots and all heart wt/body wt comparisons were performed by using StatView SE+ and ANOVA.

RESULTS

Body weight and growth. The body weight and physical state of each rat was assessed daily PI and at the time of death. No rats died or showed significant morbidity during the experimental period. All rats injected with DOX or DXR + DOX developed alopecia at the injection site on day 6 PI that had not resolved to completion at death with the largest losses found in coinjected rats, Table 1. This is in contrast to results after DOX injection into neonate day 6 rats in which alopecia was found at the head and proximal neck as well as at the site of injection (19). Cataract formation, assessed by the appearance of a white opaque eye, was noted in 1 of 20 rats in the DOX treatment group, whereas it was present in 70% of rats after injection on neonate day 6 (19). This suggests that older neonate rats are more resistant to DOX injection and that injection after eye opening is less harmful to later eye lens development.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Male Rats</th>
<th>Female Rats</th>
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<tbody>
<tr>
<td>Saline</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DXR</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DOX</td>
<td>2.43 ± 0.46</td>
<td>2.37 ± 0.55</td>
</tr>
<tr>
<td>DXR + DOX</td>
<td>4.3 ± 1.1†</td>
<td>3.66 ± 1.26‡</td>
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Values are means ± SD in centimeters squared of 8 male and 10 female rats. Animals were examined daily for hair loss at the injection site or other parts of the body. The diameter of the hair-free area of each animal was measured daily and converted to centimeters squared. Significance was calculated as †P ≤ 0.01 and ‡P < 0.001 when the dexrazoxane/doxorubicin (DXR/DOX) group was compared with the DOX injected group. Statistical analysis was performed using StatView SE+ and ANOVA.
All rats, regardless of treatment group, had similar body weights until day 3, neonate day 13 in females or day 16 PI, neonate day 26 in males. Dissection of rat pups on days 1, 5, or 9 PI did not reveal any intestinal or organ damage. This suggests that gastrointestinal lesions, potentially limiting food absorption, are unlikely to be present. In male nonexercised rats, only the DOX-alone treatment group gained significantly less weight compared with the saline nonexercised group, Table 2. Female nonexercised DOX and DXR + DOX-treated rats were significantly smaller than DXR-alone or saline-injected nonexercised controls. When treatment cohorts were compared, the body weights of nonexercised vs. exercised rats were not significantly different during the treatments or at death.

Heart wt/body wt ratios. At death, the hearts and bodies were weighed and the heart wt/body wt ratio calculated (Table 3). Nonexercised male rats treated with DXR + DOX had a significantly smaller heart wt/body wt ratio ($P < 0.05$) than the saline-treated groups. The heart wt/body wt of DXR-alone and DOX-alone groups were not significantly different from the saline-injected group. There was no significant difference in the heart wt/body wt ratios between saline and drug-injected groups in nonexercised female pups.

In males after the 2-wk exercise period, the heart wt/body wt ratios of DXR-alone and DXR + DOX groups were significantly greater ($P < 0.05$) than the saline-injected group. Within the exercised female pup groups, DXR-alone, DOX-alone, and DXR + DOX groups exhibited significantly higher ($P < 0.01$) heart wt/body wt ratios than the exercised saline-injected group.

We compared the heart wt/body wt between exercised and nonexercised rats within treatment groups (Table 3). All female exercised rats showed significantly higher heart wt/body wt ratios compared with the corresponding heart wt/body wt of the nonexercised treatment group. All drug-treated and exercised male rats had significantly greater heart wt/body wt ratios than that of the corresponding nonexercised rats. No significant increase in heart wt/body wt was found when exercised and nonexercised saline-injected male rats were compared. When the change in heart wt/body wt in male and female rats was compared within treatment groups, there was a greater increase in the heart wt/body wt with exercise in female than male rats for DXR-alone and DOX-alone ($P < 0.05$) but not DXR + DOX-treated females.

Histological results. Light microscopic examination of heart cross sections showed no difference in morphology between controls and the injected groups or between genders. No differences in cardiac morphology were noted when the exercise vs. no-exercise groups were compared. Testes and ovaries were smaller in the DOX and DXR + DOX groups than in the saline or DXR-alone-treated groups at death. However, active spermatogenesis was present in all testes, and developing follicles were present in all ovaries. Gonadal maturation present in testes and ovaries suggests the presence of relatively normal levels of circulating steroid hormones.

Protein expression. To assess whether increased apoptosis was present in hearts of drug-treated rats or was induced by swimming exercise, we measured Bcl-2, BAX, and BNIP1 protein levels, and procaspase 3 protein and its activation products on Western blots, Fig. 2. In nonexercised rats, expression of the proapoptosis marker BAX and antiapoptosis marker Bcl-2 was similar in all male and female rats. There was no change in Bcl-2 expression in exercised male rats, regardless of treatment. In contrast, Bcl-2 expression was reduced in exercised female rats in the DOX-alone (0.24 ± 0.1, $P < 0.05$) DXR-alone (0.40 ± 0.12, $P < 0.05$), and DXR + DOX (0.11 ± 0.05, $P < 0.01$) treatment groups. BNIP1 is a proapoptotic BH3-only protein. BNIP1 was increased in nonexercised female drug-treated rats (2.6 ± 0.6-fold, $P < 0.01$), but was at similar levels in all exercised female rats. Caspase 3 activation products were not present in nonexercised or exercised male rats. In female rats, caspase 3 activation products were detected in all drug-treated and nonexercised female rats and in all treatment groups in exercised female rats.

To determine whether acute oxidative stress was present, we measured heme oxygenase-1 (HO-1) protein, Fig. 3. Expression of HO-1 was similar in all treatment groups in both male and female rats nonexercised and in exercised male rats. In contrast, HO-1 expression was increased in drug-treated and exercised female rats (2.3 ± 0.6-fold, $P < 0.05$). To deter-

### Table 2. Increase in body weight of treatment groups at death

<table>
<thead>
<tr>
<th></th>
<th>Male Rats</th>
<th>Female Rats</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No exercise</td>
<td>Exercise</td>
</tr>
<tr>
<td>Saline</td>
<td>176 ± 19.6</td>
<td>187 ± 14.7</td>
</tr>
<tr>
<td>DXR</td>
<td>179 ± 14</td>
<td>202 ± 17</td>
</tr>
<tr>
<td>DOX</td>
<td>148 ± 26</td>
<td>144 ± 16</td>
</tr>
<tr>
<td>DXR + DOX</td>
<td>162 ± 12</td>
<td>155 ± 11</td>
</tr>
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</table>

Values are means ± SD in grams of 8 male and 10 female rats. Animals were weighed at death. Significance was calculated as $^*P < 0.05$, †$P < 0.01$ and ‡$P < 0.001$ when the drug-treated groups were compared with the saline-injected group. Statistical analysis was performed using StatView SE+ and ANOVA.
mine whether long-term oxidative stress was present, we measured protein carbonyl modification of heart proteins (Fig. 3). Exposure to oxidative stress causes carbonyl formation of amino acids that can be derivatized by dinitrophenol hydrazine. The level of derivatized amino acid carbonyls was unaffected by drug treatment and did not increase with exercise training in male or female rats. Clusterin is a prosurvival chaperone protein that increases as part of the unfolded protein response in oxidative-stress-induced apoptosis and is thought to preserve the integrity of critical proteins (45). Clusterin was reduced in DXR and DXR\textsubscript{H11001}DOX treatment groups in nonexercised and exercised female rats (0.34\textpm 0.17, \textit{P} \textless 0.05) and (0.52\textpm 0.1, \textit{P} \textless 0.05), respectively. In contrast, clusterin expression was not reduced in DOX-alone or DXR + DOX treatment groups in nonexercised and exercised female rats (0.34 \textpm 0.17, \textit{P} \textless 0.05) and (0.52 \textpm 0.1, \textit{P} \textless 0.05), respectively. In contrast, clusterin expression was not reduced in DOX-alone or DXR + DOX-treated nonexercised female rat samples but was reduced in DOX-alone (0.38 \textpm 0.17, \textit{P} < 0.05)-exercised male rats.

Early growth response gene-1 (Egr-1) is a transcriptional activator of several cardiac-specific genes and is increased in hearts after DOX treatment of adult mice (33). Egr-1-deficient mice respond poorly to DOX, suggesting that Egr-1 reduces DOX cardiotoxicity (35). Expression of Egr-1 was similar in all treatment groups in nonexercised male and female rats (Fig. 4). However, Egr-1 protein was reduced in exercised male rats treated with DXR-alone (0.46 \textpm 0.12, \textit{P} \textless 0.05) or DXR + DOX (0.44 \textpm 0.13, \textit{P} \textless 0.05). In exercised female rats Egr-1 expression was reduced in DXR-alone (0.08 \textpm 0.06, \textit{P} < 0.01), DOX-alone (0.40 \textpm 0.15, \textit{P} \textless 0.05), and DXR + DOX (0.16 \textpm 0.1, \textit{P} < 0.01)-treated groups.

DISCUSSION

DXR coinjection protects against DOX-induced cardiotoxicity in adult male rats and mice (7, 12, 16, 18, 35). We did not detect any cardiac damage in any drug-treated rat, exercised or nonexercised, when hearts were examined by light microscopy. We initiated a swim program to measure the ability of the
saline- and drug-treated hearts to function in response to an increase in cardiac demand. All male and female rats, regardless of treatment, completed the swim program, suggesting no incapacitating physiological deficit arose from the drug treatments. Exercised rats were not significantly different in body weight from their similarly treated nonexercised cohort. However, the heart wt/body wt of drug-treated and exercised rats was significantly increased compared with that of either their saline or nonexercised cohorts. Increases in heart wt/body wt in both genders suggests that the hearts of all drug-treated rats had to hypertrophy to accommodate the increased cardiac output necessary for adaptation to the swim program. The heart wt/body wt increase in all drug-treated and exercised rats also suggests that drug treatment did not prevent the hearts from responding with a hypertrophy response when faced with the increased demand. Furthermore, the heart wt/body wt increase in the exercised vs. nonexercised females was greater in the DOX and DXR-treated groups ($P < 0.05$) than that of the males suggesting that female hearts were more affected by the drug treatments than the males. We conclude that a functional cardiac deficit was present in drug-treated hearts and that female rats were more profoundly affected than were male rats. This is especially relevant in the absence of histological evidence of any cardiac damage in the drug-treated male and female rats. The present study clearly shows that DXR does not have a protective effect against this functional test of DOX cardiotoxicity. The results support studies (21, 30, 39, 43) that showed decreased cardiac function and a diminished exercise capacity in children exposed to anticancer cocktails that included DOX.

The apoptosis death program in the heart, like other tissues, is induced by a broad range of stimuli and is executed through activation of a series of caspases that...
include caspase 3. Our data show that swimming did not induce caspase 3 activation in control or exercised drug-treated male rats. Rats induced to exercise on a treadmill also showed no features of apoptosis (20) suggesting that moderate exercise does not provoke apoptosis in normal or DOX-treated male hearts. However, caspase 3 activation products were present in exercised and nonexercised DXR-alone, DOX-alone, and DXR + DOX-treated female rats, and were present in saline-treated exercised female rats. The caspase 3 activation is likely downstream of a decrease in the antiapoptotic protein Bcl-2 and increase in the proapoptosis protein BNIP1 in the exercised female rats. Apoptosis after a single DOX injection in cardiomyocytes can be biphasic where apoptosis is found within the first 1–2 days, becomes undetectable, and then is appreciable 3 wk later (4, 27). Thus it is not surprising that caspase 3 activation is detectable 4 wk after our drug injection in the nonexercised female rats. The iron chelators tachpyridine, dipiridyl, and desferrioxamine caused caspase 3 activation in HeLa cells (12). The presence of caspase 3 activation in the DXR-alone-injected female rat hearts, but not in similarly treated male rat hearts indicates that iron chelation-induced apoptosis occurs in the heart. Our data suggest that young female rats are more sensitive to DXR and DOX damage than young male rats.

DOX-mediated cardiac damage arises because it induces oxidative stress. Support for this mechanism is provided by studies showing increased resistance to DOX in transgenic mice that overexpress catalase in the heart (23). HO-1 is increased in acute stress, catalyzes the rate-limiting step in the oxidative degradation of heme, is increased in cardiomyocytes after incubation with hydrogen peroxide, an intermediate in DOX-induced oxidative stress and participates in the defense mechanism against oxidative stress that leads to apoptosis (6, 44). Metal oxidation of proteins produces carbonyl groups (41) and their detection provides a measure of accumulated oxidative stress. There was no change in protein carbonyl formation in any samples suggesting that oxidative stress, if present earlier, had been largely resolved to normal levels by the time of death and that this moderate amount of exercise did not cause appreciable cumulative oxidative stress. However, whereas we found no evidence for long-term oxidative stress, the increase in HO-1 protein suggests acute oxidative stress was present in female exercised rats. This increase was present in exercised female but not male rats, suggesting that this level of exercise causes a transient oxidative stress only in females. Clusterin expression is increased in heart in response to hydrogen peroxide among other inducers (24, 31). Clusterin is thought to bind critical proteins preparatory to their later refolding by heat shock proteins, and to act as a scavenger and disposal chaperone for irreparably damaged proteins and is likely important in the unfolded protein response in endoplasmic reticulum-mediated apoptosis. A reduction in clusterin levels would therefore suggest a limited or limiting mechanism(s) was available to protect damaged proteins. In females, but not males, decreased clusterin correlated with detection of caspase 3 activation products. We conclude that drug-treated female rat hearts may be more susceptible to damage and apoptosis because of a reduction in clusterin-mediated protective mechanisms and the presence of acute oxidative stress.

Estrogen can function as an antioxidant. At the time of death, male rats were approaching puberty, female rats had achieved puberty, and all rats, regardless of treatment, had histological evidence of gamete formation. This data make it improbable that either sex had sufficient testicular or ovarian damage to prevent ovarian and testicular hormone production. Thus although we think it unlikely that differential effects on steroid hormone production could account for the gender-specific effects, it remains possible that low levels of hormones, perhaps sufficient for gamete development yet insufficient for cardioprotection, were present.

Egr-1 is a transcriptional activator increased in response to diverse stimuli that includes DOX in hearts and cardiomyocytes (23, 33, 34). DOX-treated adult female mice show increased Egr-1 cardiac expression (33). The role of Egr-1 may be cardioprotective and Egr-1 may function as part of DXR-mediated cardioprotection because Egr-1-deficient mice have a more exaggerated response to DOX and their DOX-induced cardiotoxicity is not reduced by coinjection with DXR (35). Thus Egr-1 represses DOX toxicity and is involved in the cardioprotective mechanism of DXR action in adults. Young male and female rats in DXR-alone and DXR + DOX treatment groups, and also DOX-alone-treated female rats, were unable to maintain Egr-1 levels when under exercise stress. It may be significant that DXR and DOX bind iron and iron deprivation inhibited Egr-1 expression in HI-60 cells (1). Among Egr-1 responsive genes are those thought to limit cardiac damage, such as FGF-2 and SOD1 as well as those involved in calcium handling, and ultimately contraction, such as the saco(endo)plasmic reticulum Ca2+-ATPase (SERCA2) (23). The data implicate Egr-1 as limiting cardiac damage and our data suggest that previously damaged cardiomyocytes are unable to protect themselves by maintaining Egr-1 levels.

In conclusion, we found the effects of a single DOX injection into neonate male and female pups were not equivalent. An unexpected finding was that the cardioprotectant, DXR, did not suppress DOX-induced cardiotoxicity in neonate rats. Our study shows that the neonate female rat heart, has an increased sensitivity to DOX-mediated cardiac damage than does a neonate male heart, and suggests that strategies to reduce DOX-mediated toxicity in adults cannot be directly applied to the young.

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DISCLOSURES

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